Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*

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Auxin biosynthesis in plants has remained obscure although auxin has been known for decades as a key regulator for plant growth and development. Here we define the *YUC* gene family and show unequivocally that four of the 11 predicted YUC flavin monooxygenases (YUC1, YUC2, YUC4, and YUC6) play essential roles in auxin biosynthesis and plant development. The *YUC* genes are mainly expressed in meristems, young primordia, vascular tissues, and reproductive organs. Overexpression of each *YUC* gene leads to auxin overproduction, whereas disruption of a single *YUC* gene causes no obvious developmental defects. However, *yuc1yuc4*, *yuc2yuc6*, all of the triple and quadruple mutants of the four *YUC* genes, display severe defects in floral patterning, vascular formation, and other developmental processes. Furthermore, inactivation of the *YUC* genes leads to dramatically reduced expression of the auxin reporter DR5-GUS in tissues where the *YUC* genes are expressed. Moreover, the developmental defects of *yuc1yuc4* and *yuc1yuc2yuc6* are rescued by tissue-specific expression of the bacterial auxin biosynthesis gene iaaM, but not by exogenous auxin, demonstrating that spatially and temporally regulated auxin biosynthesis by the *YUC* genes is essential for the formation of floral organs and vascular tissues.

[*Keywords*: Auxin; auxin biosynthesis; flavin monooxygenase; flower development; vascular development]

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Auxin is a key regulator for many aspects of plant growth and development including floral organ formation and vascular tissue patterning, but the underlying mechanisms are not well understood. It is generally believed that cell fate and organ formation in plants are determined by local auxin gradients, which are generated by PIN family proteins that control the directions of auxin flow and overall auxin distribution (Friml et al. 2002, 2003; Benkova et al. 2003; Reinhardt et al. 2003; Heisler et al. 2005; Leyser 2005). A key missing link in our understanding of auxin-regulated developmental processes is that the molecular mechanisms of auxin biosynthesis are not known. Without knowing where auxin is synthesized in plants, it is difficult to understand the exact mechanisms of auxin movements and how auxin gradients are created and maintained.

Although multiple pathways including several tryptophan-dependent routes and a tryptophan-independent pathway are proposed to synthesize auxin in plants, the molecular details and the physiological roles of each pathway are not known (Bartel 1997; Cohen et al. 2003). The only auxin biosynthesis pathway that is well defined both biochemically and genetically is the iaaM/iaaH pathway that was found in plant pathogens such as *Pseudomonas* and *Agrobacterium*, but not in plants (Kosuge et al. 1966; Comai and Kosuge 1982). The iaaM gene encodes a tryptophan-2-monooxygenase that catalyzes the conversion of tryptophan to indole-3-acetamide, which is then hydrolyzed to release indole-3-acetic acid (IAA) by the hydrolase iaaH (Kosuge et al. 1966). Genetic dissection of auxin biosynthesis in plants has proven very difficult, and no auxin-deficient mutant has ever been identified. However, there are several auxin overproduction *Arabidopsis* mutants including *yucca* (Zhao et al. 2001), *sur1* (Boerjan et al. 1995; King et al. 1995), *sur2* (Delarue et al. 1998), and CYP79B2 overexpression lines (Zhao et al. 2002). Both SUR1 and SUR2 are not directly involved in auxin biosynthesis, and mutations in the two genes lead to the diversion of indole-3-acetaldoxime from glucosinolate synthesis to auxin biosynthesis (Barlier et al. 2000; Mikkelsen et al. 2004). Indole-3-acetaldoxime is synthesized from tryptophan by the cy-

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tochrome P450 CYP79B2/B3, which appear not to play a critical role in plant development (Zhao et al. 2002).

We previously proposed that a flavin monooxygenase, encoded by the *YUCCA1* (*YUC1*) gene, plays a key role in auxin biosynthesis through analysis of a dominant gain-of-function *yucca* mutant in *Arabidopsis* (renamed as *yuc1D* here) (Zhao et al. 2001). However, in the absence of loss-of-function mutants, it has been difficult to prove the role of *YUC1* in *Arabidopsis*. Here we define the *YUC* gene family in *Arabidopsis*, examine the expression patterns of several *YUC* family members, and use reverse genetics to unequivocally show that these YUC flavin monooxygenases are involved in auxin biosynthesis. We show that disruption of certain combinations of four of the 11 predicted *YUC* genes (*YUC1*, *YUC2*, *YUC4*, *YUC6*) (Fig. 1A) in *Arabidopsis* causes dramatic developmental defects that are rescued by the bacterial auxin biosynthesis gene iaaM under the control of a corresponding *YUC* promoter, but not by applications of exogenous auxin. Furthermore, we show that the *YUC* genes are not ubiquitously expressed and inactivation of the *YUC* genes leads to decreased expression of an auxin reporter DR5-GUS in the cells where the *YUC* genes are expressed. These data provide unambiguous evidence that the loss-of-function *yuc* phenotypes are caused by local auxin deficiency and that the YUC flavin monooxygenases are key auxin biosynthesis enzymes. More importantly, we provide evidence demonstrating that spatially and temporally regulated auxin biosynthesis is a key determinant essential for the formation of floral organs and vascular tissues, and other developmental processes. Moreover, the temporal and spatial patterns of auxin biosynthesis now can be inferred from the expression patterns of the *YUC* genes, providing a necessary foundation for analysis of auxin dynamics in plants.

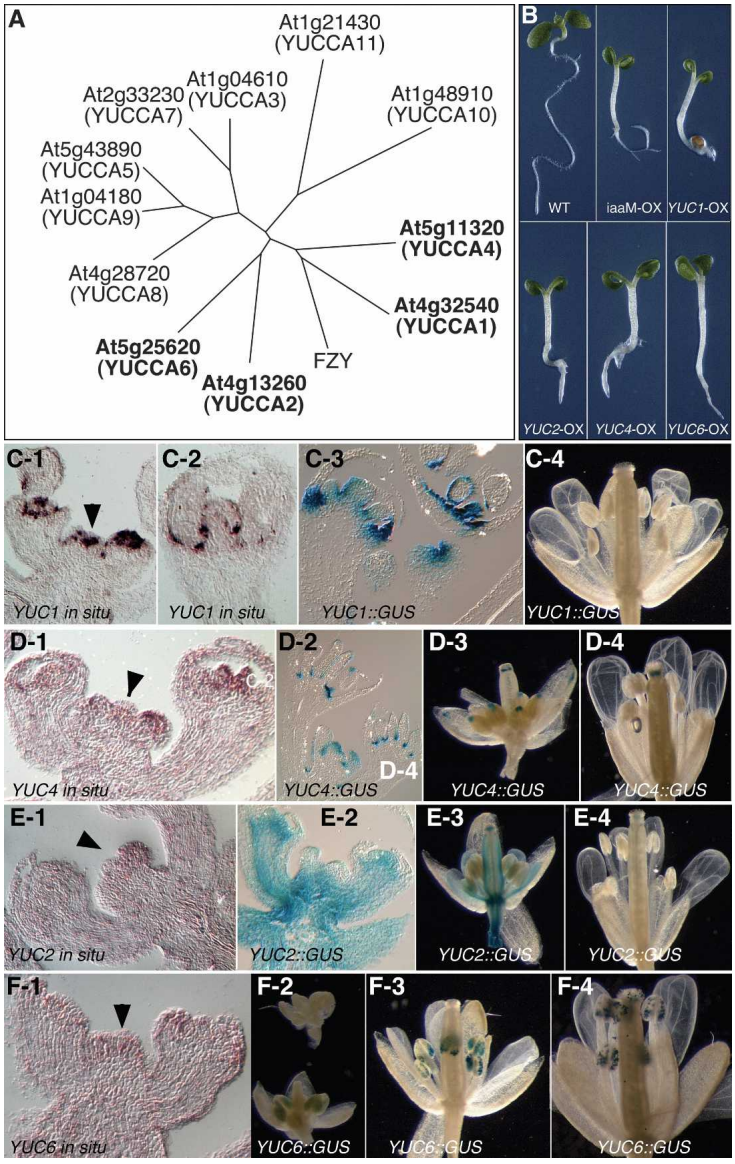

Figure 1. Overexpression of the *YUC* genes and analysis of the expression patterns of the *YUC* genes. (*A*) A phylogenetic tree of the YUC family of flavin monooxygenases. Each YUC is assigned a YUC number largely based on the order of their discoveries. The petunia YUC1 homolog FZY is also included. (*B*) Overexpression of the *YUC* genes leads to auxin overproduction. (*Top* panel from *left* to *right*) Wild type, iaaM, *YUC1*. (*Bottom* panel from *left* to *right*) *YUC2*, *YUC4*, and *YUC6*. (OX) Overexpression. (*C*) *YUC1* expression. (*C-1*,*C-2*) *YUC1* expression in the inflorescence apex and young flowers. (*C-3*,*C-4*) GUS staining of the *YUC1*::*GUS* reporter lines. (*D*) *YUC4* expression. (*D-1*) In situ hybridization of *YUC4* in the inflorescence apex. (*D-2*,*D-3*,*D-4*) GUS staining of the *YUC4*::*GUS* reporter lines. (*E*) *YUC2* expression. (*E-1*) In situ hybridization of *YUC2* in the inflorescence apex. (*E-2*–*E-4*) GUS staining of *YUC2*::*GUS* reporter lines. (*F*) *YUC6* expression. (*F-1*) In situ hybridization of *YUC6* in the inflorescence apex. (*F-2*–*F-4*) GUS staining of the *YUC6*::*GUS* reporter lines. Arrowheads indicate the center of the shoot meristem proper.

Results

Overexpression of YUC *genes leads to auxin overproduction*

The *Arabidopsis* genome contains 10 *YUC1*-like genes (Fig. 1A). In this study, we focus our analysis on the roles of *YUC1* and the three closest *YUC1* homologs (*YUC2*, *YUC4*, and *YUC6*) in auxin biosynthesis and plant development (Fig. 1A). To test whether these four *YUC* genes function in auxin biosynthesis, we constructed transgenic *Arabidopsis* plants that overexpress the cDNA of each of these *YUC* genes under the control of the Cauliflower Mosaic Virus 35S promoter. As shown in Figure 1B, overexpression of each *YUC* gene led to phenotypes indistinguishable from those of *yuc1D* and the iaaM overexpression lines, suggesting that overexpression of each *YUC* gene also caused auxin overproduction. Therefore, it is likely that the *YUC*-like genes in this study also play a role in auxin biosynthesis because of their sequence homology with *YUC1* and the observed auxin overproduction phenotypes in the overexpression lines.

Expression patterns of YUC1, YUC2, YUC4, *and* YUC6 *in the inflorescence apex and flowers*

To uncover the roles for each *YUC* gene in plant development, we analyzed the expression patterns of these four *YUC* genes by RNA in situ hybridization and by histochemistry of transgenic lines harboring promoter–GUS (β-Glucuronidase) fusions. *YUC4*, the gene with highest sequence identity to *YUC1* in *Arabidopsis* (Fig. 1A), shows expression patterns similar to that of *YUC1* in the inflorescence apex (Fig. 1C-1,D-1). Both *YUC1* and *YUC4* are expressed in the apical meristems and young floral primordia (Fig. 1C-1,D-1). Initiation of flower primordia correlates well with the accumulation of *YUC1* and *YUC4* mRNAs (Fig. 1C-1,D-1). *YUC1* is also expressed in the floral meristems and at the basal end of the young floral buds (Fig. 1C-1,C-2). It is also evident that *YUC4* is expressed in the floral meristems (Fig. 1D-1). Consistent with the in situ hybridization data, the *YUC1*::*GUS* lines showed strong staining in the floral meristems and at the base of the floral organs (Fig. 1C-3). At later stages of flower development, the GUS staining of the *YUC1*::*GUS* lines is localized in discrete groups of cells in both stamens and carpels (data not shown). In the fully matured flowers, the expression of the GUS reporter in *YUC1*::GUS lines is completely shut down (Fig. 1C-4). The *YUC4*::GUS lines also show GUS staining patterns similar to those observed from the in situ hybridization (Fig. 1D-1,D-2). Significant GUS staining was observed at the base of the floral organs in young flower buds (Fig. 1D-2). In late stages of flowers, the GUS staining of *YUC4*::*GUS* lines was found predominantly in the apical regions of the carpels, stamens, and sepals (Fig. 1D-3). In mature flowers, the GUS staining was limited only to parts of the gynoecium (Fig. 1D-4). Unlike *YUC1* and *YUC4*, *YUC2* and *YUC6* show very low expression in the inflorescence apex (Fig. 1E,F). However, weak staining was observed throughout the inflorescence apex including the stems of the *YUC2*::*GUS* lines (Fig. 1E-2). The *YUC2*::*GUS* lines also show staining in the young flower buds, petals, stamens, and gynoecium of young flowers (Fig. 1E-3). Staining in late stages of flowers in *YUC2*::*GUS* lines is mainly restricted to the valves of the gynoecium (Fig. 1E-3,E-4). Unlike the *YUC2*::*GUS* lines, the *YUC6*::*GUS* lines have staining mainly in stamens and pollen (Fig. 1F). The *YUC* genes are not ubiquitously expressed; instead, their expression is rather temporally and spatially restricted, suggesting that normal auxin functions may be dependent on the locations of auxin production.

Expression of the YUC *genes in other tissues*

The public *Arabidopsis* microarray databases (https://www.genevestigator.ethz.ch) indicate that the four *YUC* genes are also expressed in other tissues (Zimmermann et al. 2004) (Supplementary Fig. 1). Our promoter::GUS lines also showed that the *YUC* genes have distinct staining patterns in seedlings and young leaves (Supplementary Fig. 2). Strong GUS staining was observed at the base of young leaf primordia in *YUC1*::*GUS* lines, whereas GUS staining was observed in discrete spots of both basal and apical regions of young leaves in *YUC4*::*GUS* lines (Supplementary Fig. 2). We did not observe GUS staining in mature leaves in *YUC1*::*GUS* lines, but weak staining was observed in higher-order vascular strands in *YUC4*::*GUS* plants (Supplementary Fig. 2). GUS staining in *YUC2*::*GUS* lines was evident in young leaf primordia and adult leaves (Supplementary Fig. 2). No GUS staining was observed in seedlings of *YUC6*::*GUS* lines.

Identification of loss-of-function mutants for the YUC1, YUC2, YUC4, *and* YUC6 *genes*

We identified T-DNA insertion mutants for the four *YUC* genes from the T-DNA mutant collections (Fig. 2A; Tissier et al. 1999; Alonso et al. 2003) and carried out phenotypic analysis of the identified T-DNA mutants, but none of the mutants displayed any obvious developmental defects (data not shown). These observations are consistent with our hypothesis that there are overlapping functions among the *YUC* family members (Zhao et al. 2001). We did not observe any PCR products when we used primers flanking the T-DNA insertion sites to perform RT–PCR with total RNA from the *yuc* mutants (Supplementary Fig. 3).

YUC1, YUC2, YUC4, *and* YUC6 *redundantly regulate* Arabidopsis *growth and stature*

We systematically generated all six combinations of double mutants for the four *YUC* genes to test for func-

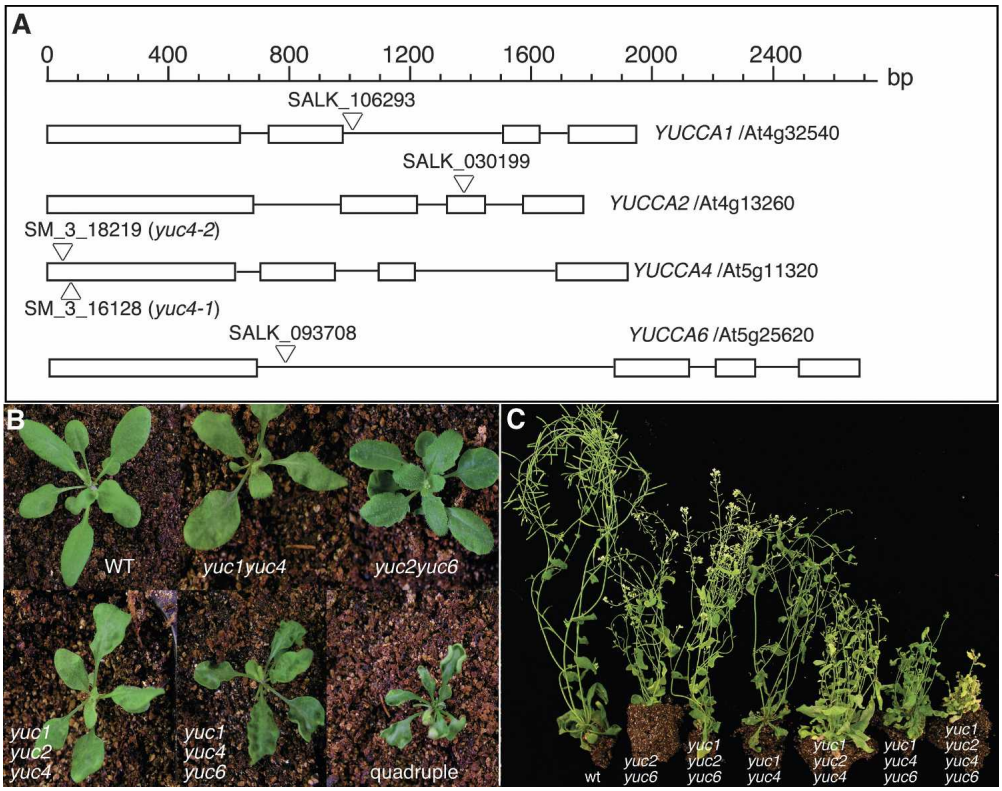

Figure 2. Analysis of the loss-of-function *yuc* mutants. (*A*) Identification of T-DNA insertion mutants for the *YUC* genes. The T-DNA insertion sites were schematically indicated and the exact insertion sites shown as the distance from the ATG codon in the genomic sequence: *yuc1*, 1027 base pairs (bp); *yuc 2*, 1348 bp; *yuc4-1*, 245 bp; *yuc4-2*, 174 bp; and *yuc 6*, 1022 bp. (*B*) Mature plants. (*Top*, *left* to *right*) Wild type, *yuc1yuc4*, and *yuc2yuc6*. Both *yuc4-1* and *yuc4-2* in various combinations with other *yuc* mutants showed the same phenotypes. The phenotypic characterizations shown in this paper are based on the *yuc4-1* allele. (*Bottom*, *left* to *right*) *yuc1yuc2yuc4*, *yuc1yuc4yuc6*, and *yuc1yuc2yuc4yuc6*. (*C*) Defects in plant stature and apical dominance. (From *left* to *right*) Wild type, *yuc2yuc6*, *yuc1yuc2yuc6*, *yuc1yuc4*, *yuc1yuc2yuc4*, *yuc1yuc4yuc6*, and *yuc1yuc2yuc4yuc6*.

tional redundancy. Among the double mutants, *yuc1yuc2*, *yuc1yuc6*, *yuc2yuc4*, and *yuc4yuc6* showed no obvious differences from wild type in terms of overall growth and development (data not shown). However, both *yuc1yuc4* and *yuc2yuc6* displayed growth and developmental defects (Fig. 2B,C). Interestingly, YUC1 and YUC4 belong to one clade, whereas YUC2 and YUC6 form another clade (Fig. 1A). This suggests that YUC1 is functionally and evolutionally closer to YUC4 and that YUC2 probably shares more overlapping functions with YUC6. Although *yuc1yuc4* and *yuc2yuc6* seedlings appeared normal, the rosette leaves of adult plants were slightly different from wild type (Fig. 2B). Both *yuc2yuc6* and *yuc1yuc4* were slightly shorter than wild type (Fig. 2C), and both double mutants showed decreased apical dominance (Fig. 2C). The *yuc1yuc4* mutant was completely sterile, whereas *yuc2yuc6* had dramatically reduced fertility (Fig. 2C).

The *yuc1yuc2yuc6* and *yuc2yuc4yuc6* triple mutants were very similar to *yuc2yuc6* double mutants (Fig. 2C; data not shown). However, *yuc1yuc2yuc4* and *yuc1yuc4yuc6* showed phenotypes much more severe than the *yuc1yuc4* double mutant (Fig. 2B,C). The *yuc1yuc2yuc4* triple mutant had rosette leaves similar to those of *yuc1yuc4*, but with even more curvatures (Fig. 2B), whereas the rosette leaves of *yuc1yuc4yuc6* were more severely affected (Fig. 2B). Compared with *yuc1yuc4*, the triple mutants displayed smaller statures and more extreme in decreased apical dominance (Fig. 2C). The y*uc1yuc2yuc4yuc6* quadruple mutant is much smaller and has much more severe phenotypes than all of the other *yuc* mutant combinations (Fig. 2B,C).

The YUC1, YUC2, YUC4, *and* YUC6 *genes have unique and overlapping functions in floral organ patterning*

Both *yuc2yuc6* and *yuc1yuc4* are essentially sterile (Fig. 2C), but the infertile phenotypes in the two mutants were caused by defects in different parts of the flowers. Wild-type *Arabidopsis* flowers have four sepals, four petals, six stamens, and two fused carpels (Fig. 3A). Although all four types of organs were present in *yuc2yuc6* flowers (Fig. 3B), their stamens were shorter than those of wild type (Fig. 3B). In addition, the anthers of *yuc2yuc6* matured later than normal and rarely produced any pollen (Fig. 3B).

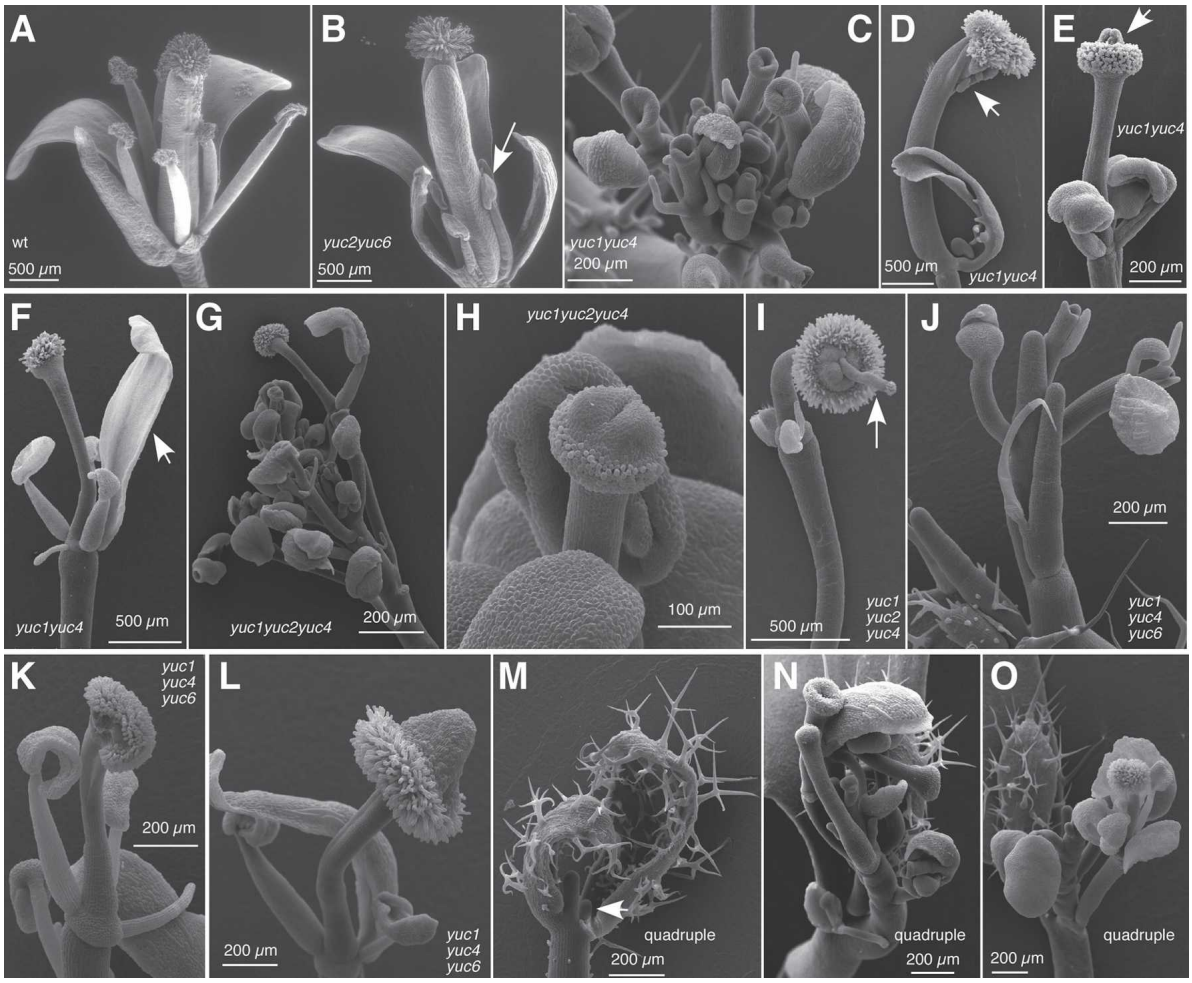

Figure 3. The roles of the *YUC* genes in floral organ patterning. (*A*) A wild-type flower with some sepals and petals removed. (B) A flower of *yuc2yuc6* with some sepals and petals removed. The arrow points to the short stamens. (*C–F*) Flower-like structures of *yuc1yuc4*. The inflorescence apex of *yuc1yuc4* is shown in *C*, and typical *yuc1yuc4* flowers are displayed in *D–F*. The arrow in *D* indicates the ovule-like structure, the arrow in *E* points to the meristem-like protrusion, and the arrow in *F* refers to a petal-like organ. (*G–I*) Flowers of *yuc1yuc2yuc4*. The inflorescence apex is shown in *G* and typical flowers are shown in *H* and *I*. In *I*, a gynoecium-like structure grew out of the *top* of another gynoecium. (*J–L*) Various floral structures of *yuc1yuc4yuc6*. (*M–O*) Inflorescence apex of *yuc1yuc2yuc4yuc6*. Note the cylinder structure in *M*.

Unlike *yuc2yuc6*, *yuc1yuc4* displayed defects in all four whorls of floral organs, and no functional reproductive organs were ever observed (Fig. 3C–F). All flowers in *yuc1yuc4* lacked certain floral organs (Supplementary Table 1), but the severity of defects varied among flowers. Some flowers had sepal-like organs (Fig. 3D), while other flowers totally lacked such sepal-like tissue (Fig. 3E). We observed stamen-like structures in some, but not all, flowers (Fig. 3D,E). The stamen-like structures never produced any pollen. We also observed petal-like tissue in some flowers (Fig. 3F). In some flowers (Fig. 3D), we observed two fused carpels with normal looking stigma tissue on the top. Occasionally, we observed ovules outside of the gynoecium (Fig. 3D). In other flowers, the stigma-like tissue formed a ring with a protrusion of meristem-like tissue in the center (Fig. 3E).

Like the *yuc1yuc4* double mutant, *yuc1yuc2yuc4* and *yuc1yuc4yuc6* also displayed floral defects in all four whorls (Fig. 3G–L). Sometimes, a gynoecium-like structure grew out of the top of another gynoecium to form a spectacular structure (Fig. 3I). Compared with the double mutants, we noticed that the triple mutants made fewer flowers, and their flower structures appeared slightly smaller than the *yuc1yuc4* double mutant.

The *yuc1yuc2yuc4yuc6* quadruple mutant had even stronger flower phenotypes than the triple mutants described above. The inflorescence meristem was often very small, and no flower buds were visible except for some cylinder-like structures at early stages of development (Fig. 3M). Overall, the flowers of the quadruple mutant were smaller than the triple mutants. It appeared that the quadruple mutant had the ability to produce every type of the floral organ, but not every flower had all types of organs. Like the double and triple mutants, we

also observed variations from flower to flower in the quadruple mutant (Fig. 3M–O).

The four YUC *genes redundantly regulate venation in flowers*

In wild-type flowers, vascular bundles form a continuous vascular network (Fig. 4A). The vascular bundle development in *yuc2yuc6* flowers appeared normal (Fig. 4B). In the gynoecium-like structures of *yuc1yuc4* flowers, the major veins are missing, and the observed vascular bundles were largely not connected to each other (Fig. 4C). The *yuc1yuc2yuc4* and *yuc1yuc4yuc6* triple mutants had fewer vascular bundles than the *yuc1yuc4* double mutant, and in some organs there were no vascular bundles at all (Fig. 4D,E). In most cases, no veins were seen in the gynoecium-like structures in the quadruple mutant, and no vascular bundles were observed in the floral stems (Fig. 4F). It is clear that the four *YUC* genes are important for floral vascular development, but *YUC1* and *YUC4* play a more prominent role than *YUC2* and *YUC6*.

The four YUC *genes are necessary for vascular development in leaves*

In *Arabidopsis* rosette leaves, the vascular tissue is composed of a primary vein in the middle of a leaf; secondary veins originate from and connect to the primary vein, multiple tertiary veins, and higher-order veins (Fig. 4G). Overall, the vascular tissues in the *yuc2yuc6* double mutant appeared to be normal (Fig. 4H). The *yuc1yuc4* double mutant had fewer veins in leaves than wild type (Fig. 4I). Very few quaternary and higher-order veins presented in *yuc1yuc4* (Fig. 4I). When *YUC2* was inactivated in the *yuc1yuc4* background, it caused a greater decrease in vein numbers (Fig. 4J). Not only were quaternary veins missing, but the *yuc1yuc2yuc4* triple mutant also had fewer tertiary veins (Fig. 4J). When *yuc1yuc4* was combined with *yuc6*, the triple mutant had even fewer secondary veins (Fig. 4K), but the veins along the edge of the leaf still formed a continuous circle. The quadruple mutant had fewer veins than the triple mutant, and the veins along the edge of the leaf no longer formed a continuous circle (Fig. 4L). The observed gradient in phenotypic strength in terms of vein numbers and continuity of the vascular strands among the *yuc* mutants strongly indicates that certain thresholds of local auxin concentrations, which are dependent on the *YUC* genes, have to be reached in order for vascular tissue formation, demonstrating that locally produced auxin is an important determinant for vascular strand formation.

Disruption of the YUC *genes leads to decreased expression of the auxin reporter DR5-GUS*

DR5-GUS has been widely used as an auxin reporter (Sabatini et al. 1999). In the wild-type background, GUS staining in DR5-GUS plants is observed in root tips, cotyledon tips, and newly formed leaves (Fig. 5A). The DR5-GUS-staining patterns in wild-type background presented here are the same as those previously documented (Mattsson et al. 2003). In the *yuc1yuc2yuc6* mutant background, GUS staining in root tips and cotyledon tips is similar to that in wild type in terms of GUS-staining pattern and intensity (Fig. 5B). However, GUS staining in young leaves of *yuc1yuc2yuc6* was much reduced (Fig. 5B). In the *yuc1yuc4yuc6* background, we also observed much reduced GUS staining in young leaves, whereas staining in roots and cotyledons appeared unchanged (Fig. 5C). The reduced DR5-GUS staining was mainly observed in tissues where the *YUC* genes are expressed (Fig. 5A–C; Supplementary Fig. 2).

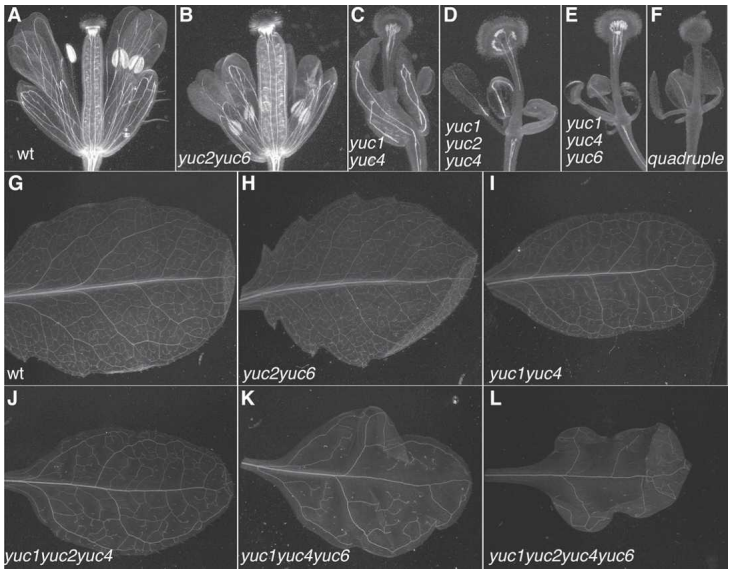

Figure 4. Venation patterns in *yuc* flowers and leaves. (*A–F*) Flower venation patterns. (*G–L*) Vascular patterns in leaves. (*A*,*G*) Wild type. (*B*,*H*) *yuc2yuc6*. (*C*,*I*) *yuc1yuc4*. (*D*,*J*) *yuc1yuc2yuc4*. (*E*,*K*) *yuc1yuc4yuc6*. (*F*,*L*) *yuc1yuc2yuc4yuc6*.

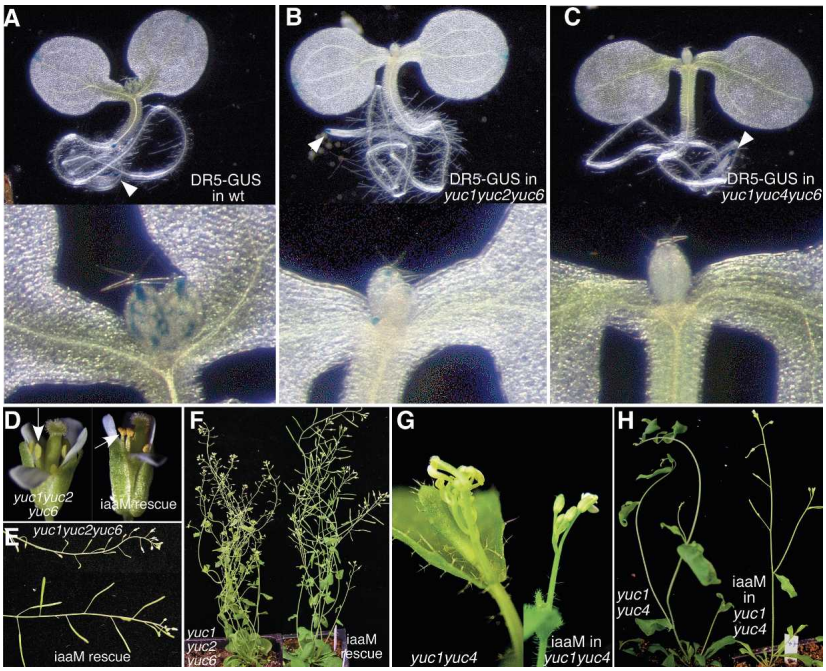

Figure 5. Effects of *yuc* mutations on the expression of DR5-GUS and complementation of *yuc* mutants with the iaaM gene. (*A*) DR5-GUS in wild-type background. (*B*) DR5-GUS in *yuc1yuc2yuc6* background. (*C*) DR5-GUS in *yuc1yuc4yuc6* background. (*A–C*) The *top* shows the whole seedling, and the *bottom* shows the true leaves. Arrowheads point to root tips. (*D*) A flower of *yuc1yuc2yuc6* (*left*) and a flower of *yuc1yuc2yuc6* transformed with the iaaM gene under the control of the *YUC6* promoter (*right*). Note the differences in stamens (arrows). (*E*) The infertile phenotypes of *yuc1yuc2yuc6* (*top*) were rescued by the expression of the iaaM gene (*bottom*). (*F*) The decreased apical dominance phenotypes of *yuc1yuc2yuc6* (*left*) were complemented by the iaaM gene. (*G*) Complementation of the floral defects of *yuc1yuc4* (*left*) by expressing the iaaM gene under the control of the *YUC1* promoter (*right*). (*H*) The sterile phenotypes of *yuc1yuc4* (*left*) were rescued by the iaaM gene (*right*).

Developmental defects of yuc1yuc2yuc6 *and* yuc1yuc4 *are rescued by the bacterial auxin biosynthesis gene iaaM*

To further test whether the observed developmental defects in the various loss-of-function *yuc* mutants were caused by auxin deficiency, we tried to rescue the *yuc* loss-of-function phenotypes by applying exogenous IAA (20 and 100 μM) to the mutants, but repeated applications of exogenous auxin failed to complement either *yuc1yuc4* or *yuc2yuc6* or other *yuc* mutants. It is possible that exogenous auxin may not be up-taken efficiently and accumulated to the right concentrations in cells. Therefore, we decided to mimic *YUC*-directed auxin production in vivo by expressing the bacterial auxin biosynthesis gene iaaM under the control of a *YUC* promoter. Previous studies have clearly shown that iaaM converts tryptophan to auxin once it is expressed in plants (Klee et al. 1987; Romano et al. 1995). If the phenotypes of the *yuc* mutants are caused by auxin deficiency, the in situ production of auxin by the iaaM gene should rescue the mutant defects. We transformed a segregating population with the iaaM gene under the control of the *YUC6* promoter. The segregating population was the self-fertilized offspring from plants that were *yuc1* homozygous and *yuc2yuc6* heterozygous. Among the 71 T1 plants analyzed, seven plants were identified as *yuc1yuc2yuc6* that also contained the iaaM gene. Like *yuc2yuc6* shown above (Figs. 2C, 3B), the *yuc1yuc2yuc6* triple mutant had short stamens and rarely produced any pollen (Fig. 5D), which led to essentially sterile phenotypes (Fig. 5E). The overall plant structure and stature of the iaaM transgenic plants looked like wild type. More importantly, the stamens developed normally in the iaaM transgenic lines and produced normal amounts of pollen (Fig. 5D). Furthermore, expression of the iaaM gene also rescued the infertility (Fig. 5E) and the decreased apical dominance phenotypes (Fig. 5F). The *yuc1yuc2yuc6* phenotypes were also rescued by expressing the iaaM gene under the control of the *YUC2* promoter (data not shown).

We also tested whether the iaaM gene can rescue the *yuc1yuc4* double mutant, which displayed much more severe floral defects than the *yuc1yuc2yuc6* triple mutant (Fig. 3). The *yuc1yuc4* double mutant never produces any functional reproductive floral organs and is totally sterile (Figs. 2, 3). As shown in Figure 5G, expression of the iaaM gene under the control of a *YUC1* promoter rescued the overall floral defects of *yuc1yuc4*, and the rescued plants produced functional pollen and carpel to set seeds (Fig. 5H). We never observed meristem-like tissue on top of the gynoecium in the rescued flowers, whereas such tissue is common in the *yuc1yuc4* flowers (Fig. 3; Supplementary Table 1). Unlike the *yuc1yuc4* flowers that never produced the right number or the normal structure of sepals, petals, and stamens (Fig. 3; Supplementary Table 1), the rescued flowers always had four sepals (Supplementary Table 2). In the rescued flowers, the structures of petals and stamens are normal looking, but the numbers varied among flowers (Supplementary Table 2). These results suggest that the amount of auxin produced by the heterologous enzyme is still not optimal. We also discovered that *YUC6*::*iaaM* could not rescue *yuc1yuc4* (data not shown).

The iaaM rescue experiments clearly demonstrate that the developmental defects in *yuc1yuc4* and *yuc1yuc2yuc6* are caused by tissue-specific auxin deficiency and the YUC flavin monooxygenases are key enzymes for auxin biosynthesis. Our findings thus show that normal auxin functions depend on the source of auxin biosynthesis,

demonstrating that regulated auxin biosynthesis by YUC flavin monooxygenases plays essential roles in *Arabidopsis* development.

Discussion

Auxin biosynthesis by the YUC flavin monooxygenases

Our genetic studies using both gain-of-function and loss-of-function mutants of the *YUC* family genes clearly demonstrate the important roles of the *YUC* family of flavin monooxygenase genes in auxin biosynthesis. We and others have clearly shown that overexpression of *YUC1* and *YUC-like* genes in *Arabidopsis* and petunia causes auxin overproduction (Fig. 1B; Zhao et al. 2001; Marsch-Martinez et al. 2002; Tobena-Santamaria et al. 2002; Woodward et al. 2005). Like the *yuc1/floozy* mutant in petunia, disruption of the *YUC* genes in *Arabidopsis* also leads to defects in floral development, vascular tissue formation, and other developmental processes, indicating the essential roles of the *YUC* genes in plant growth and development (Figs. 2–4). We further demonstrated that inactivation of *YUC* genes in *Arabidopsis* led to reduction of GUS staining of the auxin reporter DR5-GUS (Fig. 5). Furthermore, the developmental phenotypes of the *yuc* mutants were rescued by expression of the bacterial auxin biosynthesis gene iaaM under the control of a corresponding *YUC* promoter (Fig. 5), providing unambiguous evidence that the *YUC* genes play a key role in auxin biosynthesis and plant development.

We have shown that the *YUC* genes have unique and overlapping expression patterns and their expression is temporally and spatially regulated. It becomes apparent that the location of auxin biosynthesis is critical for plant development, as we observed that different combinations of the *yuc* mutants display different phenotypes (Figs. 2–4). For example, the *yuc2yuc6* double mutant has defects mainly in stamen development, whereas inactivation of both *YUC1* and *YUC4* affected the number and identity of floral organs (Fig. 3). Furthermore, we showed that the auxin reporter DR5-GUS was regulated by the *YUC* genes in a tissue-specific manner, demonstrating that the *YUC* genes provide a mechanism to produce auxin with temporal and spatial precision.

Genetic redundancy in auxin biosynthesis

It is evident that the four *YUC* genes (*YUC1*, *YUC2*, *YUC4*, and *YUC6*) have overlapping functions. Although the quadruple mutant had very severe developmental defects, it was not seedling or embryo lethal. Therefore we suspect that additional *YUC* genes have overlapping functions with the four *YUC* genes shown in this study. We and others have shown that at least two other *YUC* genes (*YUC3* and *YUC5*) (Fig. 1A) are probably also involved in auxin biosynthesis because they caused the same phenotypes as those of *yuc1D* when they were overexpressed in *Arabidopsis* (Woodward et al. 2005; Y. Zhao, Y. Cheng, and X. Dai, unpubl.). In addition, it is known that auxin can be synthesized through the CYP79B2/B3 pathway and the tryptophan-independent pathway (Zhao et al. 2002). Construction of combinations of quintuple and sextuple *yuc* mutants or even higher-order mutants will be informative in defining the roles of each *YUC* gene in other developmental processes.

It is not understood when the duplications of the *YUC* genes occurred during plant species evolution. *YUC* genes have been found in other plant genomes including rice, maize, medicago, and petunia. Interestingly, genetic redundancy of the *YUC* genes was less complex in petunia, because inactivation of the *YUC1* ortholog in petunia led to phenotypes similar to *yuc1yuc4* in *Arabidopsis* (Tobena-Santamaria et al. 2002). Analysis of *YUC* genes in other plant species may provide clues as to how vascular plants may have evolved and how auxin may play a role during that process.

Auxin and flower development

Previous studies demonstrated that auxin signaling or polar transport mutants including *pin1, pinoid, ettin,* and *mp* have abnormal flowers, suggesting that auxin plays a critical role in flower development (Okada et al. 1991; Berleth and Jurgens 1993; Bennett et al. 1995; Sessions and Zambryski 1995). The observed floral phenotypes in the *yuc* mutants were very similar in many ways to the flowers of *pin1*, *pinoid,* and *ettin* (Okada et al. 1991; Bennett et al. 1995; Sessions et al. 1997). One similarity between the *yuc* flowers and the *pin1/pinoid* flowers is the huge variation from flower to flower in these mutants (Fig. 3). In *pin1* mutants, some flowers do not have stamens, but have petals with abnormal shapes and variable numbers (Okada et al. 1991). Some *pin1* flowers have a pistil-like structure without any sepals, petals, and stamens (Okada et al. 1991). Flowers of *pinoid* also displayed a wide range of variations (Bennett et al. 1995). As shown in Figure 3, flowers of *yuc1yuc4*, two triple mutants, and the quadruple mutant also had great variation. Some flowers of *yuc1yuc4* were very similar to those *pin1* flowers with pistil-like structures (Fig. 3D). The quadruple mutant has pin-like structures in the inflorescence apex (Fig. 3). Another similarity between the *yuc* mutants and the known auxin mutants (*pin1*, *pinoid*, and *ettin*) is that all four whorls of floral organs are affected in these mutants, indicating that auxin probably affects floral meristem. The phenotypic similarities in flowers between the *yuc* mutants and the known auxin mutants further support the notion that the *YUC* genes affect an auxin-related process, which we have shown to be auxin biosynthesis. It will be interesting to analyze mutants that combine the *yuc* mutants with auxin transport mutants or signaling mutants. We observed that treatment of certain *yuc* mutant combinations with the auxin transport inhibitor NPA totally blocked new leaf formation, a phenotype that is not observed in the *yuc* mutants alone or NPA treatment alone (Y. Cheng and Y. Zhao, unpubl.), suggesting that plant

development is regulated by coordinated auxin biosynthesis and transport.

Unlike previous auxin mutants that only show floral organ identity defects, the *yuc* mutants appeared also to have the floral indeterminacy problem (Fig. 3). We often observed meristem-like tissue on the top of a gynoecium-like structure, where normally stigmatic tissue occurs (Fig. 3E,H,I,L). Occasionally, another gynoecium-like structure developed from the top of a gynoecium to produce the unprecedented floral structure (Fig. 3I), suggesting that auxin not only plays a critical role in specifying floral organ identities, but also may regulate floral meristemic cell proliferation.

Auxin and vascular tissue development

Auxin has long been suggested to play a critical role in vascular patterning. Auxin was shown to be sufficient to induce the formation of vascular strands from parenchymatic cells (Jacobs 1952), and overproduction of auxin in a whole plant increased amounts of vascular tissue (Klee et al. 1987). However, it has not been demonstrated whether auxin is also necessary for vascular tissue formation, although mutants in auxin signaling and polar transport displayed various vascular defects (Galweiler et al. 1998; Christensen et al. 2000). Compared with other auxin mutants such as *pin1* and *mp*, the vascular defects in the *yuc1yuc2yuc4yuc6* quadruple mutant were much more severe (Fig. 4). The number of veins, vascular continuity, and vascular patterns were all affected in the quadruple mutant (Fig. 4), demonstrating that auxin is essential for both vascular tissue formation and patterning.

Directional auxin flow has been suggested as a key determinant in vascular differentiation and patterning in the "canalization hypothesis" (Sachs 1981). The observed gradient in phenotypic strength in terms of vein numbers and continuity of the vascular strands among the *yuc* mutants strongly suggests that locally produced auxin is also an important determinant for vascular strand formation. The strong correlation between *YUC* gene dosage and the number of veins in leaves and flowers indicates that certain thresholds of auxin concentrations have to be reached in order for vascular tissue to form. The strong vascular phenotypes of the *yuc* mutants and the ordered reduction of vein numbers when *YUC* genes are successively compromised provides a great system for further analyzing the vascular pattern mechanism.

In summary, we have shown clearly that the *YUC* genes are involved in auxin biosynthesis. The *YUC* genes control several aspects of plant growth and development including the formation of floral organs and vascular tissues by producing auxin with temporal and spatial specifications.

Materials and methods

Materials

The T-DNA insertion mutants were obtained from either the *Arabidopsis* stock center at Ohio or from Dr. Joseph Ecker's laboratory at the Salk Institute. The T-DNA mutants were genotyped as described in Tissier et al. (1999) and Alonso et al. (2003). The gene-specific primers for genotyping the T-DNA mutants were as follows: *yuc1*: 5′-GGTTCATGTGTTGCCAAGGGA-3′ and 5′-CCTGAAGCCAAGTAGGCACGTT-3′; *yuc2*: 5′-CTGCATACAATCCGCTTTCGC-3′ and 5′-TTCTTGCATTTTCTCGCTCTACG-3′; *yuc4-1 and yuc4-2*: 5′-CCCTTCTTAGACCTACTCTAC-3′ and 5′-GCCCAACGTAGAATTAGCAAG-3′; *yuc6*: 5′-CCAGCCTTTGTATTTTCCCGT-3′ and 5′-CCGGAAAAAGGGTTCTTGTCG-3′.

GUS assay and histological analysis

YUC promoters were cloned into a pBI101.3 vector, and the constructs were transformed into wild-type *Arabidopsis* Columbia by floral dipping (Clough and Bent 1998). The obtained transgenic plants were used for analysis of the expression patterns of each *YUC* gene. GUS assays were as described (Zhao et al. 2001). For whole-mount analysis of vascular structures, plant tissue was prepared as described (Berleth and Jurgens 1993), and viewed/photographed under dark-field illumination in a Leica dissecting microscope. Samples for SEM analysis were prepared according to methods described in Dinneny et al. (2004). The in situ hybridization was carried out as described in Dinneny et al. (2004).

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